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Reduction of polysaccharide production in *Pseudomonas aeruginosa* biofilms by bismuth dimercaprol (BisBAL) treatment

Ching-Tsan Huang[a]* and Philip S. Stewart[b]

[a]*Department of Agricultural Chemistry, National Taiwan University, 1 Roosevelt Road Sec. 4, Taipei 10617, Taiwan, ROC;* [b]*Center for Biofilm Engineering and Department of Chemical Engineering, Montana State University, Bozeman, MT 59717, USA*

Microorganisms in biofilms, cells attached to a surface and embedded in secreted insoluble extracellular polymers, are recalcitrant to chemical biocides and antibiotics. When *Pseudomonas aeruginosa* ERC1 biofilms were treated continuously with 1 × MIC of bismuth dimercaprol (BisBAL), biofilm density determined by both total cell counts and viable cell counts increased during the first 30 h period then decreased thereafter. After 120 h of treatment there was an approximate 3-log reduction in viable cell areal density compared with the untreated control. Per-cell total polysaccharide production was significantly reduced in biofilms exposed to 12.5 μM BisBAL compared with the untreated control. In biofilm cultures, 1 × MIC of BisBAL did not initially kill attached cells but was enough to reduce polysaccharide production. As treatment proceeded, the normalized polysaccharide content was reduced and those cells attached became susceptible to 1 × MIC of BisBAL.

Introduction

Modern medical and surgical therapy is predicated on the use of catheters and endoprostheses. However, over half of hospital-acquired infections are associated with implants or indwelling medical devices. Frequently the design and preparation of biomedical materials for implantation is contradictory. While surface roughness, porosity or chemical pretreatment of the prosthesis may render an implant more biocompatible, such approaches also promote bacterial adhesion and consequently the formation of biologically active biofilms. Biofilms consist of cells and their secreted insoluble extracellular polymers, which are largely polysaccharides. They are recalcitrant to chemical biocides and to antibiotics. The development of biofilm infections on indwelling devices may lead to prolonged hospitalization, device malfunction or even mortality.

The principal strategy for managing biofilm infections relies on antimicrobial agents to kill the attached microorganisms and/or remove them from the surface. However, biofilm cultures are found to be much more difficult to eradicate than their counterparts in suspended cultures.[1–3]

An alternative approach to controlling biofilm formation could be to inhibit production of the biofilm matrix material. Recently, Cammarota and Sant'Anna[4] reported that 2,4-dinitrophenol could block extracellular polysaccharide (EPS) synthesis and consequently reduce biofilm accumulation. Another promising agent for inhibition of EPS production is a bismuth compound. In a series of papers, Domenico *et al.*[5–9] report that several bismuth compounds could reduce EPS production by Gram-negative bacteria in suspended cultures. Among these compounds, bismuth dimercaprol (BisBAL) was found effective against most bacteria.[9] However, all of these results were obtained from suspended cultures. We report in this article the application of BisBAL to reduce the total polysaccharide production in biofilm cultures.

Materials and methods

Strain and medium

An environmental isolate of *Pseudomonas aeruginosa* ERC1 was obtained from the culture collection at the Center for Biofilm Engineering, Montana State University (Bozeman, MT, USA). A defined medium (Table) containing 20 mg/L glucose as the sole carbon source was used to grow biofilms.

*Corresponding author. Tel: +886-2-2363-4796; Fax: +886-2-8773-4556; E-mail: cthuang@ccms.ntu.edu.tw

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Table. Composition of medium used in biofilm culture

Glucose	20 mg/L	$FeSO_4 \cdot 7H_2O$	159 μg/L
KNO_3	13.6 mg/L	$ZnSO_4 \cdot 7H_2O$	142 μg/L
$MgSO_4$	1.0 mg/L	$MnSO_4 \cdot H_2O$	11.4 μg/L
$CaCO_3$	1.0 mg/L	$CuSO_4 \cdot 5H_2O$	2.8 μg/L
Na_2HPO_4	426 mg/L	$Co(NO_3)_2 \cdot H_2O$	2.3 μg/L
KH_2PO_4	205 mg/L	$(NH_4)_6Mo_7O_{24} \cdot 5H_2O$	1.4 μg/L
$(HOCOCH_2)_3N$	200 μg/L	$Na_2B_4O_7 \cdot 10H_2O$	1.4 μg/L

Minimal inhibitory concentration (MIC) of BisBAL

BisBAL, kindly provided by Dr Philip Domenico (Winthrop University Hospital, Mineola, NY, USA), was prepared by dissolving bismuth nitrate in dimercaprol (BAL, Sigma Chemical Comp., St Louis, MO, USA) containing solution at a molar ratio of 1:1.6. The concentration of bismuth was used in this study to represent BisBAL concentration. The MIC of BisBAL was determined by the tube dilution method. About 10^7 cells/mL were inoculated into tubes containing medium and varying BisBAL concentrations, and shaken at 200 rpm at 35°C for 18 h.

Biofilm formation system

The experimental apparatus used for biofilm formation and its operating conditions have been described previously.[10] Briefly, biofilms were grown on removable stainless steel slides using a continuous flow annular reactor (Bio/Surfaces Technology Inc., Bozeman, MT, USA). The characteristics of the annular reactor have been described in detail elsewhere.[11,12] The reactor was operated in batch mode for 24 h to allow suspended cells to attach to stainless steel slide surfaces. Sterile medium was then fed continuously into the reactor to effect a dilution rate of 3.2/h to limit suspended cell growth. Slides with accumulated biofilms were withdrawn periodically and scraped into 50 mL phosphate buffered solution (pH 7.2) for analysis.

BisBAL treatment of biofilms

A pulse of concentrated BisBAL was added to the reactor to effect 1 × MIC BisBAL at the end of batch phase. The pulse was accompanied by continuous feeding of concentrated BisBAL to maintain the above concentration.

Analytical methods

After homogenization, biofilm suspensions were suitably diluted and plated on R2A agar (Difco, Detroit, MI, USA) plates. The number of viable cells was determined by averaging the cfu on three plates. Total cell count was obtained by acridine orange direct count using an Olympus BH-2 fluorescent microscope (Olympus, Japan). The phenol–sulphuric acid method[13] was used in the assay of total polysaccharides. Bacterial alginate from *P. aeruginosa* (Sigma Chemical Comp., St Louis, MO, USA) was used as a total polysaccharide standard.

Statistical analysis

The experiment was repeated three times. Cell and plate counts were $\log_{10}$ transformed and the means and standard errors of the means were calculated. Statistical analyses were performed using S-Plus software (Version 3.1 by Statistic Science, Inc., Seattle, WA, USA) and were based upon the log-transformed means.

Results

MIC of BisBAL

The concentrations of BisBAL tested in the MIC assay were 0, 1.56, 3.13, 6.25, 9.38, 12.50, 18.75, 25 and 37.50 μM. The results of these experiments are illustrated in Figure 1. For concentrations <12.5 μM, the viable cell concentrations can increase at least 1-log while cell concentrations for those treated with concentrations >12.5 μM did not change significantly. Therefore, 12.5 μM was determined as the MIC of BisBAL for *P. aeruginosa* ERC1. This concentration is consistent with that reported by Domenico *et al.*[7] who showed that the MIC of BisBAL for a variety of bacteria ranged from 5 to 15 μM.

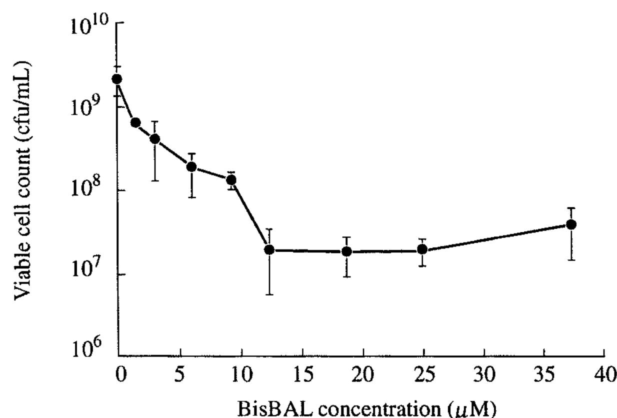

Figure 1. Determination of MIC of BisBAL for *P. aeruginosa* ERC1. ($n = 3$; bar indicates S.E.).

Bactericidal effect of BisBAL against biofilms

The decreases in *P. aeruginosa* ERC1 biofilm density determined by total cell count and viable cells in response to 12.5 μM BisBAL treatment are shown in Figure 2a and b, respectively. For the control experiment, both total cell counts and viable cell counts increase significantly during the first 72 h period then maintained a steady state thereafter. For the BisBAL-treated biofilm, total cell count increased with time initially but started to decrease after 54 h of treatment. There was a *c.* 1-log reduction before the total cell count stopped decreasing. Viable cell counts increased with time after 30 h of BisBAL treatment, followed by a dramatic reduction for the next 60 h period. A *c.* 3-log reduction was observed in comparison with the untreated control at 120 h.

Since cell numbers on each stainless steel slide might vary, the surviving fraction was used to represent the bactericidal effects of BisBAL. The surviving fraction was obtained by dividing the number of viable cells by the number of total cells. Figure 3 shows the bactericidal effects of BisBAL against *P. aeruginosa* ERC1 biofilms. For those without BisBAL treatment, the surviving fraction generally remained above 70%. The low values in surviving fraction at 6 and 30 h were not expected and might result from either underestimation of viable counts or overestimation of total cell counts. When exposed to 12.5 μM BisBAL, the surviving fraction decreased with time and there was a *c.* 1.5-log reduction at the end of experiment. The difference in surviving fraction between control and treated experiments after 120 h was statistically significant ($P < 0.05$).

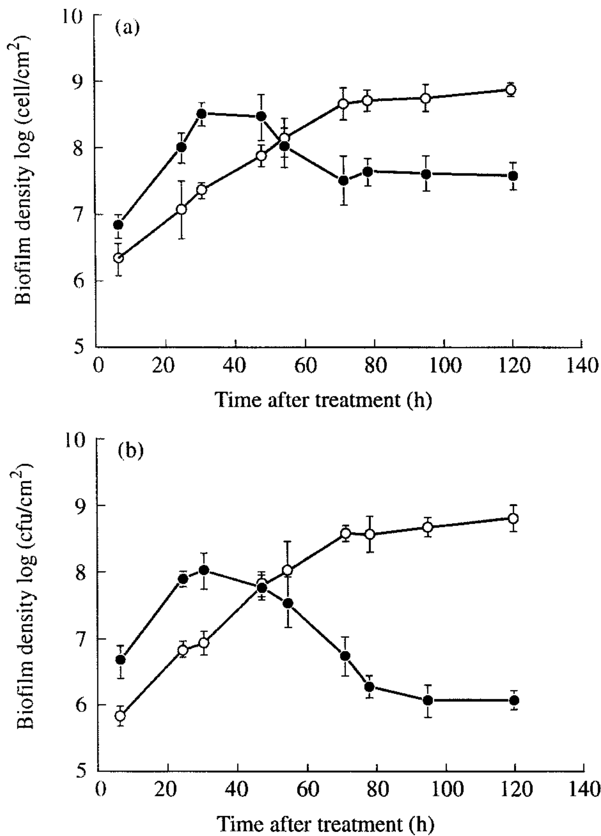

Figure 2. *P. aeruginosa* ERC1 biofilm density determined by (a) total cell counts and (b) viable cell counts in response to 12.5 μM BisBAL treatment. (○) Control; (●) treated. (n = 3; bar indicates S.E.).

Polysaccharide reduction by BisBAL treatment

The total polysaccharide production for *P. aeruginosa* ERC1 with and without BisBAL treatment is presented in Figure 4. For the control experiment, the total polysaccharide increased with time and reached an alginate equivalent of approximately 23 mg/cm^2 after 120 h of accumulation. With exposure to 12.5 μM BisBAL, the total polysaccharide increased for the first 30 h, then continued to decrease and at the end of the experiment an alginate equivalent of only 0.7 mg/cm^2 remained. To eliminate the bias due to biofilm accumulation on each slide, total polysaccharide production was normalized by total cell count.

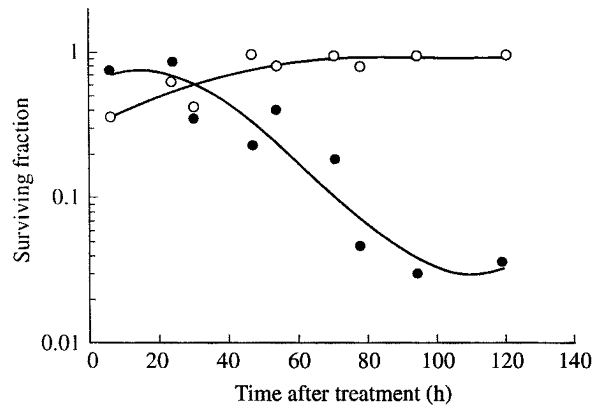

Figure 3. Surviving fraction of *P. aeruginosa* ERC1 biofilm in response to 12.5 μM BisBAL treatment. (○) Control; (●) treated.

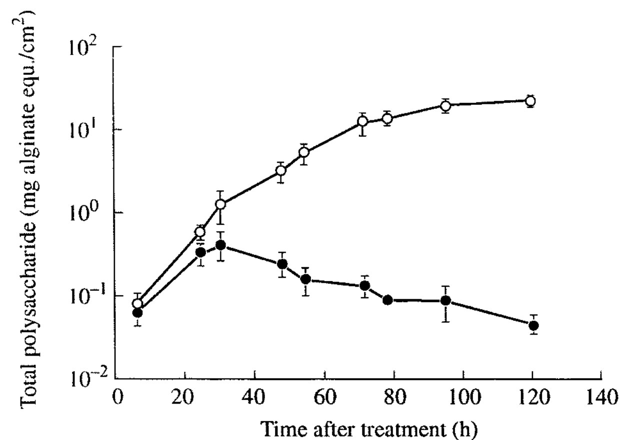

Figure 4. Total polysaccharide production of *P. aeruginosa* ERC1 biofilm in response to 12.5 μM BisBAL treatment. (○) Control; (●) treated. (n = 3; bar indicates S.E.).

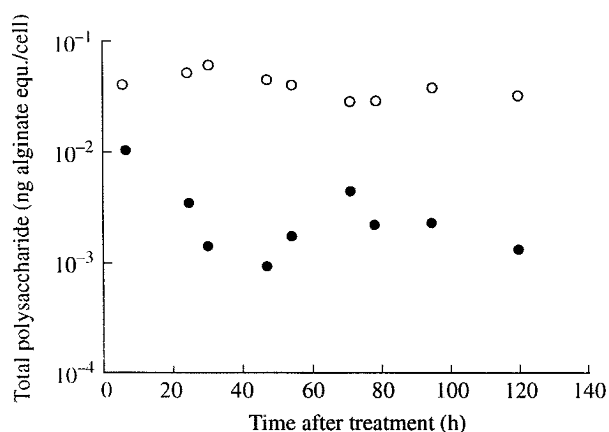

Figure 5. Normalized total polysaccharide production of *P. aeruginosa* ERC1 biofilm in response to 12.5 μM BisBAL treatment. (○) Control; (●) treated.

Figure 5 shows the total per-cell polysaccharide production of *P. aeruginosa* ERC1 biofilms in response to BisBAL treatment. The per-cell total polysaccharide was quite consistent for the control experiment, while it varied considerably for the BisBAL-treated biofilm. Nevertheless, the normalized polysaccharide content was clearly lower in the treated biofilm compared with the untreated biofilm. Statistical analysis showed that the difference in total polysaccharide per cell between control and treated cultures was significant ($P < 0.05$).

Discussion

Biofilms are known for their recalcitrance to antimicrobial treatment. Transport limitation and physiological adaptation are often mentioned to explain this enhanced resistance in bacteria within biofilms. Transport limitation is attributed to the neutralization of the antimicrobial agent in the biofilm more quickly than it can diffuse in.[14,16] In many cases, antimicrobial agents are either adsorbed by or react with the extracellular polymers, which are primarily polysaccharides. The second explanation for biofilm resistance to chemical challenge is physiological differences between biofilm and planktonic cells.[3] Microorganisms grown under phosphate or nitrogen starvation tend to produce more extracellular polysaccharides[17,18] and such starving cells in the interior of the biofilm are also candidates for reduced susceptibility.[19,20] Both mechanisms based upon transport limitation or physiological adaptation suggest that the extracellular polysaccharides play an important role in the biofilm resistance.

In this study, BisBAL was tested against *P. aeruginosa* ERC1 biofilms. Unlike most antimicrobial agents, which usually require many times the concentration needed to show antimicrobial activity against planktonic cultures to achieve similar bactericidal results against biofilm cultures, BisBAL at a concentration of $1 \times$ MIC led to a >1-log reduction in surviving fraction and an obvious reduction in polysaccharide production. For experiments operated in planktonic cultures, Domenico and co-workers have demonstrated that bismuth compounds inhibited capsular polysaccharide expression by *Klebsiella pneumoniae* and *P. aeruginosa,* at sub-MIC levels.[5,7,8] In biofilm cultures, $1 \times$ MIC of BisBAL was not sufficient to kill attached cells as observed in the first 30 h of treatment (Figure 2) but it was enough to reduce the polysaccharide production (Figure 5). As the treatment proceeded, the per-cell polysaccharide was reduced to some extent and those cells attached became susceptible to $1 \times$ MIC of BisBAL. One possible explanation of this phenomenon is that BisBAL might be adsorbed by biofilms. Consequently, the local BisBAL concentration might be higher than $1 \times$ MIC and lead to bactericidal effects. Although the details of extracellular polysaccharide inhibition by BisBAL are not fully understood, this agent may provide a useful approach in biofilm control. Bismuth compounds in combination with rifampicin or gentamicin showed enhanced activity against gastrointestinal bacterial pathogens.[21]

In conclusion, BisBAL reduced the total polysaccharide production of *P. aeruginosa* ERC1 biofilms and consequently enhanced its bactericidal effects. This result indicates that further efforts to develop agents that block polysaccharide production may be valuable in the battle to control biofilm infections.

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